PATENT SPECIFICATION

785,525

Date of Application and filing Complete Specification: May 30, 1956.

No. 16676/56.

Application made in United States of America on June 3, 1955.

Complete Specification Published: Oct. 30, 1957.

Index at acceptance:—Class 2(3), C2B37(A3: L).

International Classification:—C07d.

COMPLETE SPECIFICATION

Process for preparing a Glucosamine

ERRATA

SPECIFICATION No. 785,525

Page 2, line 71, *for* "(1945)" *read* "(1954)"

Page 4, lines 31—32, *for* "Man-ville" *read* "Mans-ville"

Page 4, line 103, *for* "miaxture" *read* "mixture"

Page 4, line 104, *for* "elating" *read* "eluting"

Page 4, line 110, *for* "actyl" *read* "acetyl"

THE PATENT OFFICE,

12*th December*, 1957.

No. 2122, then as *L. bifidus var. Penn*, and more recently as *Lactobacillus bifidus* var. *pennsylvanicus*. A strain of this microorganism is available from the American Type Culture Collection, where it is on deposit as No. 11,863. This microorganism has been known for some time to constitute an important part of the intestinal flora of a breast-fed infant.

The specific growth-promoting factor for *Lactobaccillus bifidus*, and its importance in infant nutrition, are fully discussed with reference to the growth and proliferation of the *Lactobacillus bifidus* microorganism in the intestinal tract of the young child, in United States Specification No. 2,770,573.

In that United States Patent Specification there is described and claimed the process of preparing substances which have high activity for promoting growth of *Lactobacillus bifidus* var. Penn. which comprises reacting lactose and N-acetyl-D-glucosamine in the presence of a lactase, specifically an enzyme derived from *Lactobacillus bifidus* var. Penn. That which is useful in the process.

Our assignors have now found that if the enzymatic synthesis is carried out using living intact cells of said *Lactobacillus bifidus* var. *pennsylvanicus* microorganism instead of an enzyme derived therefrom, not only is the yield of the desired microbiologically-active compound, 4-O-β-D-galactopyranosyl - N-acetyl-D-glucosamine, greater than that secured by the process of the said United States Patent Specification No. 2770573 but the product is almost all the desired active compound, practically none of the inactive isomer being present in the reaction mixture. This is a very important advantage since approximately one-half of the product secured when the enzyme is used in the reaction is not active for promoting the growth of *Lactobacillus bifidus* var. *pennsylvanicus*. By this improved method, utilising living intact cells of the microorganism itself, only negligible amounts of the inactive isomeric 6-O-β-D-galactoside

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COMPLETE SPECIFICATION

Process for preparing a Glucosamine

We, AMERICAN HOME PRODUCTS CORPORATION, a corporation organized and existing under the laws of the State of Delaware, United States of America, of 22, East 40th Street, in the City, County and State of New York, United States of America (assignee of PAUL GYOERGY and FRIEDRICH ZILLIKEN), do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to a process for preparing a glucosamine, specifically 4-O-β-D-galactopyranosyl - N - acetyl-D-glucosamine, which is active in promoting growth of *Lactobacillus bifidus* var. *pennsylvanicus* A.T.C.C. No. 11,863.

It has recently been discovered that a specific growth-promoting factor which is present in human (breast) milk is essential to promote the growth of a specific strain of the *Lactobacillus bifidus* microorganism, this specific strain being originally identified as No. 212A, then as *L. bifidus var.* Penn, and more recently as *Lactobacillus bifidus* var. *pennsylvanicus*. A strain of this microorganism is available from the American Type Culture Collection, where it is on deposit as No. 11,863. This microorganism has been known for some time to constitute an important part of the intestinal flora of a breast-fed infant.

The specific growth-promoting factor for *Lactobaccillus bifidus*, and its importance in infant nutrition, are fully discussed with reference to the growth and proliferation of the *Lactobacillus bifidus* microorganism in the intestinal tract of the young child, in United States Specification No. 2,770,573.

In that United States Patent Specification there is described and claimed the process of preparing substances which have high activity for promoting growth of *Lactobacillus bifidus* var. Penn. which comprises reacting lactose and N-acetyl-D-glucosamine in the presence of a lactase, specifically an enzyme derived from *Lactobacillus bifidus* var. Penn. That process results in a mixed product containing two chemical compounds, one of which is active in promoting growth of said microorganism, while the other is inactive. These two chemical compounds can be characterised as 4-O-β-D-galactopyranosyl - N - acetyl-D-glucosamine (active) and 6-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine (inactive).

In the process for producing these two compounds in admixture disclosed in the said United States Patent Specification No. 2770573, lactose and N-acetyl-D-glucosamine are reacted in the presence of an enzyme which is present in growing cultures of *Lactobacillus bifidus* var. *pennsylvanicus*, which enzyme is secured for use in said synthesis by a procedure described in that application. This procedure, in brief, involves centrifuging a growing culture of said microorganism, lyophilising the solid to secure a dry product, and intimately mixing the dry product with aluminium oxide in the presence of a phosphate buffer solution, thereby securing a solution of said enzyme which is useful in the process.

Our assignors have now found that if the enzymatic synthesis is carried out using living intact cells of said *Lactobacillus bifidus* var. *pennsylvanicus* microorganism instead of an enzyme derived therefrom, not only is the yield of the desired microbiologically-active compound, 4-O-β-D-galactopyranosyl - N-acetyl-D-glucosamine, greater than that secured by the process of the said United States Patent Specification No. 2770573 but the product is almost all the desired active compound, practically none of the inactive isomer being present in the reaction mixture. This is a very important advantage since approximately one-half of the product secured when the enzyme is used in the reaction is not active for promoting the growth of *Lactobacillus bifidus* var. *pennsylvanicus*. By this improved method, utilising living intact cells of the microorganism itself, only negligible amounts of the inactive isomeric 6-O-β-D-galactoside

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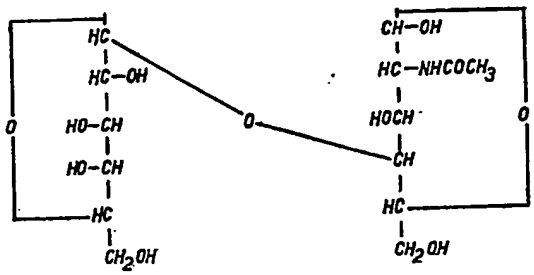
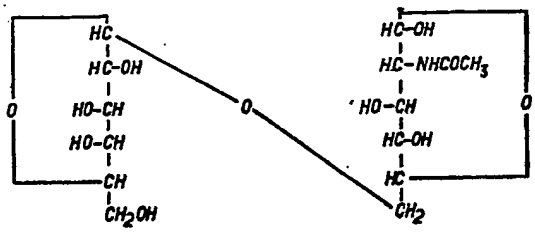
are formed. These isomeric compounds may be represented by the following structural formulae:



4-O-β-D-galactopyranosyl-N-Acetyl-D-glucosamine (Active)



6-O-β-D-galactopyranosyl-N-Acetyl-D-glucosamine (Inactive)

According to the present invention, therefore, the process of preparing 4-O-β-D-galacto-pyranosyl - N-acetyl - D-glucosamine, said compound being active in promoting growth of *Lactobacillus bifidus* var. *pennsylvanicus* A.T.C.C. No. 11863, comprises reacting lactose and N-acetyl-D-glucosamine in the presence of living intact cells of *Lactobacillus bifidus* var. *pennsylvanicus* microorganism.

This improved method wherein the reaction between lactose and N-acetyl-D-glucosamine is carried out by incubating intact cells of the microorganism with the reactants also has the further advantage that there is no loss of active disaccharide on prolonged incubation. A process which permits production of the active 4-O-β-D - galactopyranosyl - N-acetyl-D-glucosamine for periods up to 48 hours without appreciable loss of formed active product has definite commercial advantages, since it avoids the necessity, characteristic of many enzymatic methods, of stopping the reaction and recovering the product at a definite point in the synthesis.

In carrying out the improved process the lactose and N-acetyl - D-glucosamine are brought together in the presence of intact cells of *Lactobacillus bifidus* var. *pennsylvanicus*, A.T.C.C. No. 11,863, and incubation is continued for any desired period up to forty-eight hours or even longer. The greatest yield of the microbiologically-active 4-O-β-D - galactopyranosyl - N - acetyl - D-glucosamine is obtained under anaerobic conditions, but aerobic conditions can also be employed with, however, a slight increase in the amount of unwanted inactive isomer. The pH should fall within the range 4.0 to 7.0, and the temperature within the range 15° to 40° C. Very satisfactory results have been secured when the reaction is carried out at a temperature of approximately 37° C. and at a pH of approximately 5.4, the pH being maintained within the specified range by the use of a phosphate buffer.

The living microorganism may be grown on a medium containing all nutrients including the *Lactobacillus bifidus* growth-promoting factor, which are necessary for its growth and propagation. A suitable medium, to which breast milk or some other material supplying the *Lactobacillus bifidus* growth-promoting factor should be added, is disclosed in British Patent Specification No. 746,834.

It contains, in addition, to some substance supplying the growth-promoting factor and mineral salts, additional substances such as potassium acid phosphate, lactose, sodium acetate, hydrolysed casein, various amino acids, thiamine, riboflavin, calcium pantothenate, and various other vitamins. See also the paper of Gyoergy et al., *Arch. Biochem. and Biophys.*, *48* 193, (1945). The living cells may be collected by centrifugation or other suitable procedure, washed and supplied to the mixture of lactose and N-acetyl-D-glucosamine as a concentrated suspension. The reaction requires a molar ratio of lactose to N-acetyl-D-glucosamine of 2:1, and the reactants should be present in approximately this proportion. The process is advantageously carried out by suspending the intact cells of *L. bifidus* var. *pennsylvanicus* in an aqueous phosphate buffer solution, and then adding lactose and N-acetyl-D-glucosamine thereto in the molar ratio of two moles of

lactose to one of the N-acetyl-D-glucosamine. The mixture is then incubated at a temperature within the range 15° to 40° C., preferably at about 37° C. Anaerobic incubation is preferred since it results in the highest yield of the desired 4-O-β-D-galactopyranosyl - N - acetyl - D-glucosamine and minimal amounts of the active isomer. However, the incubation can also be carried out under aerobic conditions, but with a lowered yield of the desired active compound and increased amounts (although still relatively small) of the inactive isomer. The conversion of part of the lactose into undesired products such as glucose and galactose is more pronounced under aerobic conditions than when the synthesis is carried out anaerobically, but fairly good yields of 4-O-β-D-galactopyranosyl-N-acetyl-D-glucosamine can also be obtained by incubation under aerobic conditions. By anaerobic conditions we mean those in which the amount of oxygen in the ambient atmosphere in contact with the reaction mixture does not exceed 4%.

In recovering the active product, 4-O-β-D-galactopyranosyl - N acetyl-D-glucosamine, from the reaction mixture we have found it advantageous first to heat the digest, after termination of incubation, to about 100° C. for about one hour, and then centrifuge off the supernatant. The supernatant is then concentrated to a reduced volume and adsorbed on a chromatographic column containing a suitable adsorbent. This may, for example, consist of two parts of carbon and one part of diatomaceous earth. Of course other adsorbents may also be utilised, and they need not necessarily be employed in columnar form. The adsorbed material is then eluted from the column with suitable eluting agents such as water and aqueous ethanol. Evaporation of the aqueous ethanolic eluates to dryness yields a solid product comprising the active compound. It may be recovered in purified crystalline form by recrystallising it from suitable solvents, such as dry methanol, in the usual manner.

The following examples are illustrative of preferred forms of the process of the present invention.

EXAMPLE 1.

Lactobacillus bifidus var. *pennsylvanicus*, A.T.C.C. No. 11,863, was grown in accordance with the procedure described in the publication of Gyoergy, Norris and Rose, *Arch. Biochem. and Biophys.*, Vol. 48, (1954), page 193. This medium contained, as the basic medium, the mineral salts and additional substances referred to above, and also included a material supplying the essential growth factor for *L. bifidus* which may be a small amount of skimmed human milk. Cells from 40-hour cultures were collected by centrifugation and then washed twice with 0.85% saline and once with M/15 phosphate buffer of pH 5.4. A suitable phosphate buffer may comprise a 0.1 molar solution of a mixture of sodium and potassium phosphates, such, for example, as disodium orthophosphate Na_2HPO_4 and dipotassium orthophosphate K_2HPO_4.

The cells were then suspended in the same phosphate buffer solution of M/15 molar concentration so that 3 millilitres of the cell suspension represented cells from 100 millilitres of the culture. Three millilitre aliquots of this cell suspension were then added to 10 millilitre portions of M/15 phosphate buffer containing 382 milligrams of N-acetyl-D-glucosamine and 625 milligrams of lactose (monohydrate). These cell-substrate suspensions were then incubated at 37° C. in suitable cotton-plugged vessels. Anaerobic incubation was carried out by first evacuating the vessels and then filling them three times with nitrogen gas, followed by evacuation before finally restoring atmospheric pressure by the addition of a mixture comprising 10 per cent. of carbon dioxide and 90 per cent. of nitrogen. In carrying out the reaction under aerobic incubation, the flasks were allowed to stand in the atmosphere without agitation. In each case incubation was continued for periods up to 48 hours. Samples were taken at intervals and analysed for reducing sugars and N-acetylamino sugars by paper chromatography using in the test the supernatant secured by heating each sample for 10 minutes at 100° C., cooling, and centrifuging.

The greatest yields of 4-O-β-D-galactopyranosyl - N - acetyl - D-glucosamine were obtained under conditions of anaerobic incubation. Under aerobic conditions the yield was somewhat reduced and the amount of galactopyranosyl-N-acetyl - D-glucosamine somewhat larger. However, in all cases, the amount of inactive material was very small being negligible under anaerobic incubation conditions.

Formation of the active disaccharide during incubation was indicated by the regular increase in growth-promoting activity for *Lactobacillus bifidus* var. *pennsylvanicus* as expressed in growth units per millilitre of the supernatant fluid assayed. From an initial activity of 6 units per millilitre (due solely to the N-acetyl - D-glucosamine present), within 24 hours under conditions of anaerobic incubation the activity increased to 30 units per millilitre and remained at this figure throughout the entire 48 hour incubation period. Under aerobic conditions the activity at the end of 24 hours incubation had increased to 20 units per millilitre. After 48 hours of aerobic incubation the activity had dropped only slightly below the 20 units per millilitre value.

The concentration of the intact cells of *L. bifidus* var. *pennsylvanicus* in the reaction mixture did not affect production of the

active compound unduly, as the same amount of growth-promoting activity was secured when the cell concentration was only 30 per cent. of that initially tested. Synthesis occurred even when the cell concentration was only about 1 per cent. of that initially used.

EXAMPLE 2.

Lactobacillus bifidus var. *pennsylvanicus* cells were secured from 8 litres of culture medium and prepared as described in Example 1. These cells were then incubated for 24 hours in a liquid medium containing 16.5 grams of lactose (monohydrate) and 10.1 grams of N-acetyl-d-glucosamine in 345 millilitres of M/15 phosphate buffer at pH 5.4. The phosphate buffer was a mixture of disodium orthophosphate and dipotassium orthophosphate of approximately 0.1 molar concentration.

The digest was heated for one hour in an oven at 100° C. and then centrifuged. The supernatant was concentrated at a reduced pressure less than atmospheric to a volume of approximately 50 millilitres. This concentrate was adsorbed on a column comprising 320 grams of decolourising carbon and 160 grams of diatomaceous earth. For the decolourising carbon it was found convenient to use Norite A (Pfanstiehl) and the diatomaceous earth was Celite 535 (Johns-Manville Corporation). ("Norite" and "Cellite" are Registered Trade Marks).

The column was then eluted with water and aqueous ethanol, the general procedure followed being that described in the paper by Zilliken, Smith, Rose and Gyoergy in *Journal Biol. Chem.*, *208*, page 299 (1954).

The eluates of the 7.5% ethanol concentration were evaporated to dryness at a reduced pressure less than atmospheric. The amorphous residue was dried under potassium pentoxide in a desiccator and then dissolved in 80 millilitres of hot dry methanol. The hot methanolic solution was then filtered and allowed to stand at room temperature. Crystallisation of square platelets began immediate and was completed after a few hours standing at 5° C. After recrystallisation of the product from a minimum amount of dry methanol there was obtained 1.02 grams of the disaccharide, the yield being approximately 5.4%. A sample of the product had a melting point of 172° C. (uncorrected); a specific rotation of $[\alpha]_D^{25} + 27.8°$ (H_2O;C=1, equilibrium rotation reached after 180 minutes). Extrapolated to zero time, the rotation was $[\alpha]_D + 51.2°$. The disaccharide crystallised with one mole of methanol and analysis confirmed the empiric formula $C_{14}H_{25}O_{11}N.CH_3OH$. Comparison of the product by the mixed melting point method, and by microbiological assay, with pure 4-O-β-D - galactopyranosyl - N - acetyl - D-glucosamine as obtained from hog stomach mucin showed that the product was identical with the known compound as secured from animal tissue.

The product was added as such and without further purification both to foodstuffs which were deficient in, and to foodstuffs which were totally lacking in, the *L. bifidus* growth-promoting factor. In this way the resulting food products were analogous to human milk in that they contained this important nutritional factor.

What we claim is:—

1. The process of preparing 4-O-β-D-galacto-pyranosyl - N-acetyl - D-glucosamine, said compound being active in promoting growth of *Lactobacillus bifidus* var. *pennsylvanicus* A.T.C.C. No. 11,863, which comprises reacting lactose and N-acetyl-D-glucosamine in the presence of living intact cells of said *Lactobacillus bifidus* var. *pennsylvanicus* microorganism.

2. The process as claimed in claim 1 in which the reaction is carried out at a pH within the range 4.0 to 7.0 and at a temperature within the range 15° to 40° C.

3. The process as claimed in claim 2 in which the reaction is carried out at a temperature of approximately 37° C. and a pH of 5.4.

4. The process as claimed in claim 3 wherein said pH is maintained by the presence in said reaction mixture, together with said reactants, of a phosphate buffer capable of maintaining said specified pH.

5. The process as claimed in any of claims 1 to 4 in which the 4-O-β-D-galacto-pyranosyl-N-acetyl-D-glucosamine is isolated by adsorbing the reaction miaxture on a chromatographic column, elating therefrom a solution containing the compound to be isolated, and crystallising such compound from anhydrous methanol.

6. The process of preparing 4-O-β-D-galacto-pyranosyl-N-acetyl-D-glucosamine as herein particularly described.

7. 4-O-β-D-galacto - pyranosyl-N-actyl-D-glucosamine, whenever prepared by the process claimed in any of the preceding claims.

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